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The interaction between drought and elevated CO_2 in water relations in two grassland species is species-specific

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ABSTRACT

Climate change can have major consequences for grassland communities since the different species of the community utilize different mechanisms for adaptation to drought and elevated CO_2 levels. In addition, contradictory data exist when the combined effects of elevated CO_2 and drought are analyzed because the soil water content is not usually similar between CO_2 concentrations. Thus, the objectives of this work have been to examine the effect of water stress on plant water relations in two grassland species (*Trifolium pratense* and *Agrostis capillaris*), analyzing the possible differences between the two species when soil water content is equal in all treatments, and to elucidate if development under elevated CO_2 increases drought tolerance and if so, which are the underlying mechanisms. At ambient CO_2, when soil volumetric water content was 15%, both species decreased their water potential in order to continue taking up water. *Trifolium pratense* performed osmotic adjustment, while *Agrostis capillaris* decreased the rigidity of its cell wall; moreover, both species increased the root to shoot ratio and decreased leaf area. However, these mechanisms were not sufficient to maintain cell turgor. Elevated CO_2 partially mitigated the negative impact of drought on turgor potential in *Trifolium pratense* through a higher osmotic adjustment and root to shoot ratio and in *Agrostis capillaris* through a higher leaf relative water content caused by higher hydraulic conductance, but the impact of drought was not mitigated in either species by higher soil water conservation.

1. Introduction

The atmospheric CO_2 concentration has increased since the beginning of the industrial revolution, from a concentration of 280 μmol mol^{-1} to the current concentration of 400 μmol mol^{-1} (Stocker et al., 2011), mainly due to anthropogenic causes. Recently, the IPCC (2014) stated that the atmospheric concentration of CO_2 increases at an average rate of 2.0 ± 0.1 μmol mol^{-1} $year^{-1}$, the largest average increase since direct CO_2 concentration measurements began in 1958. On this basis, the IPCC (2014) has predicted several scenarios regarding the future climate conditions; in this respect, the average estimate for the concentration of CO_2 in the atmosphere at the end of the century would be approximately 700 μmol mol^{-1}, *i.e.*, twice that registered at the beginning of the same century.

In addition to increasing the concentration of CO_2, climate change is expected to provoke a change in the amount of rainfall, increasing in some areas and significantly decreasing in others. In addition, due to the change in the distribution of rainfall, extreme events of drought and flooding are expected to increase (IPCC, 2014). These global climatic changes can have major consequences for terrestrial vegetation, including short-term physiological responses in crop plants and long-term changes in the structure and function of ecosystems (Mooney et al., 1991; Smith et al., 2005; Naudts et al., 2011, 2013; Peñuelas et al., 2013; Xu et al., 2014).

Water is the most limiting resource for plant growth (Boyer, 1982; Chaves et al., 2003), and its scarcity jeopardizes the productivity of crops and grasslands. Drought alters the aeration and hydraulic conductance of the soil, causing a decrease in soil water potential (Rozema,

Abbreviations: Ac, *Agrostis capillaris*; DH, dehydration; DW, dry weight; FW, fresh weight; HC, hydraulic conductance; Ψ_{opd}, leaf predawn osmotic potential; Ψ_{ppd}, leaf predawn turgor potential; Ψ_{wpd}, leaf predawn water potential; Ψ_{omd}, leaf midday osmotic potential; Ψ_{pmd}, leaf midday turgor potential; Ψ_{wmd}, leaf midday water potential; Ψ_o^{100}, leaf osmotic potential at full turgor; OA, osmotic adjustment; Ψ_o, osmotic potential; RWC, relative water content; θv, soil volumetric water content; Tp, *Trifolium pratense*; TW, turgid weight; Ψ_p, turgor potential; ε, volumetric elasticity modulus; Ψ_w, water potential

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1993), which complicates water uptake. Therefore, plants must be able to tolerate or adapt to drought as a mechanism to sustain their development and productivity. Drought tolerance is a complex trait involving multiple adaptations. The key to this is that the plant must have the ability to maintain water uptake in increasingly dry soil while minimizing water loss through its leaves. Moreover, differences among species are also observed, as each species has different mechanisms for adaptation to drought (Xu et al., 2014; AbdElgawad et al., 2015).

Plants achieve this tolerance by means of morphological adaptations such as the development of more numerous and/or longer roots or alterations in leaf morphology, reducing their size and/or increasing the cuticle, and by physiological and biochemical adaptations such as stomatal closure to prevent water loss, and decreasing water potential (Ψ_w) to maintain water uptake. Thus, several authors have described that some plants are able to maintain water uptake by actively decreasing Ψ_w through the lowering of osmotic potential (Ψ_o). Ψ_o is determined by the concentration of osmotically active substances, especially in the vacuole, and depends on the degree of hydration, the absolute amount of osmolytes, and the relative distribution of water between the symplast and apoplast fractions (Girma and Krieg, 1992; Sánchez-Blanco et al., 1998). A decrease in Ψ_o may occur either by dehydration (DH), which causes a passive increase in the concentration of osmolytes, or by active synthesis of osmolytes, which is known as osmotic adjustment (OA) (Martìnez et al., 2004; Martínez et al., 2007; Pérez-López et al.,2010). OA includes the active accumulation of organic and inorganic solutes in the cell, thereby achieving a reduction of Ψ_o and an increase in the inflow of water to the cell, maintaining turgor pressure (Sánchez et al., 2004).

Nevertheless, the usefulness of OA as a mechanism of drought tolerance is controversial. Some authors have suggested that OA is a result of reduced growth by drought (Munns, 1988). Others have suggested that OA only operates under severe water deficit, when the plant's survival is threatened (Serraj and Sinclair, 2002).

In the case that dehydration occurs, this cellular water loss may result in a loss of turgor. To maintain turgor despite the lower water content, plant adjusts their cell wall elasticity, making it more elastic (Saito and Terashima, 2004). However, the opposite trend has also been observed, *i.e.*, the plant decreases the elasticity, making it more rigid (Martínez et al., 2007) to reduce Ψ_w with minimal loss of leaf water and, therefore, maintain water uptake. Both strategies are considered advantageous for the plant (Schulte, 1992; Saito and Terashima, 2004).

As mentioned above, another mechanism for tolerating drought is to diminish water loss. To achieve this goal and, therefore, maintain a better water status, plants tend to reduce transpiration by stomatal closure, by reducing hydraulic conductance and/or by decreasing leaf area (Sadras et al., 1993; Liu and Stützel, 2002; Robredo et al., 2007).

In addition to increased soil drought, as aforementioned, the future climatic conditions are expected to provoke an increase in the atmospheric concentration of CO_2, which under well-watered conditions has not generally affected water relations (De Luis et al., 1999; Robredo et al., 2007; Perry et al., 2013). However, when the increase in the concentration of CO_2 acts together with drought, interactive effects have been detected in the response of different morphological, physiological and biochemical parameters (Xu et al., 2013 and references therein). Regarding water relations, several studies have shown that plants have a better water status than at ambient CO_2 due to reduced transpiration rate as a result of the stomatal closure associated with increased CO_2 (Robredo et al., 2007; Leakey et al., 2009; Morgan et al., 2011; Miranda-Apodaca et al., 2015). Conversely, other studies have not observed differences between CO_2 concentrations (Ferris and Taylor, 1994; Manderscheid et al., 2014), and in some studies, the effect of increased CO_2 concentration on water status is even negative (Centritto et al., 1999; De Luis et al., 1999; Zeppel et al., 2012). Furthermore, Samarakoon and Gifford (1995) established that the response of transpiration to the combined effects of elevated CO_2 and drought is determined by the relationship between the increase in leaf area and the decrease in transpiration per area induced by stomatal closure. Thus, if the decrease in transpiration per area is greater than the increase in leaf area, the total transpiration will be reduced; however, if the increase in leaf area is greater the total transpiration will increase.

In addition, some authors have argued that, under elevated CO_2, the increased CO_2 assimilation rate would permit the plant to have more carbon skeletons to perform OA (Wall et al., 2006; Chen et al., 2015); however, other authors have observed that due to the higher growth rate at elevated CO_2 the concentration of solutes in the leaf could decrease, turning Ψ_o less negative and observing a minor OA (Tschaplinski et al., 1993; 1995). Furthermore, some authors have affirmed that elevated CO_2 would permit the plant to increase the fine roots and, in general, the root biomass, raising the root to shoot ratio, boosting drought tolerance (Xu et al., 2013). Thus, there is not a general consensus regarding how the interaction between drought and elevated CO_2 affects water relations and associated tolerance mechanisms; probably because in the aforementioned studies the water content of the soil was not always similar between CO_2 treatments, which could be masking direct and specific effects of elevated CO_2.

Grasslands are one of the largest ecosystems, covering approximately 25% of land cover (FAO, 2010) and constituting approximately 40% of the potential agricultural area in Western Europe (Peeters, 2004). Furthermore, due to its wide occurrence, it is argued that the grassland ecosystem is of great importance as a potential mitigating factor for climate change through underground carbon sequestration; FAO (2010) established that the total carbon sequestration by grasslands is 50% higher than that by woods.

With all this in mind, the objectives of this work have been to examine the effect of water stress on plant water relations in two grassland species (*Trifolium pratense* and *Agrostis capillaris*), analyzing the possible differences between the two species when soil water content is equal in all treatments and to elucidate whether development under elevated CO_2 increases drought tolerance and if so, which are the underlying mechanisms. With these objectives, the hypotheses were 1) when all treatments have the same amount of water in the soil, the species that can develop more efficient mechanisms of drought tolerance will present greater turgor potential and leaf water content; 2) under combined environmental conditions, the elevated CO_2 will partially mitigate the negative effect of drought, through higher root to shoot ratio and osmotic adjustment as a consequence of the increased availability of carbon skeletons for use in the synthesis of compatible osmolytes, and not through higher soil water conservation.

2. Materials and methods

2.1. Plant material and experimental design

Two grassland species were used: *Agrostis capillaris* L. (Ac; grass) and *Trifolium pratense* L. (Tp; legume). Seedlings were grown in a mixture of peat/vermiculite (1/1 v/v) in 6 L pots. Thirty-six seeds were sown in each pot and were watered every other day with deionized water for 4 days till the plants germinated. Fourteen days after sowing, the twelve more uniform plants were selected, reaching a final density of 315 plant m^{-2}. After emergence and for 24 days, the seedlings were watered two days a week with 250 mL of Hoagland's solution, and in order to reach field capacity, the plants were also watered with deionized water between each application of Hoagland's solution.

Plants were grown in a Conviron PGR15 controlled environment growth chamber (Conviron, Manitoba, Canada) with a daily 14 h light regime, an average day/night temperature of 24/20 °C, and a relative day/night humidity of 70/80%. During the light period, the photosynthetic photon flux density in the chamber was 400 μmol m^{-2} s^{-1}. Light was provided by a combination of incandescent bulbs and warm-white fluorescent lamps.

The CO_2 treatments were 400 μmol mol^{-1} (ambient CO_2) and 700 μmol mol^{-1} (elevated CO_2). The growth chamber atmosphere was

maintained at ambient or elevated CO_2 from sowing throughout growth to the end of the experiment. The CO_2 concentration was monitored by the software of the Conviron PGR15 chamber.

The application of drought treatment started 4 weeks after sowing and lasted 16 days. The implementation of drought marked day 0 of the experiment and began with all the pots at field capacity. The drought treatment was imposed by withholding water until the soil volumetric water content (θv) reached 15% of the value measured at field capacity, which was calculated from the daily difference in weight of the pots (volume of water) divided by the volume of the pots (volume of soil). From that day on, the pots were watered daily with the amount needed to keep the θv at 15%. In the well-watered plants, the θv was held at 100%, watering daily. Also during this treatment period, the watering was alternated daily between Hoagland's solution and deionized water.

2.2. Water relations

The leaf relative water content (RWC) was measured by gravimetric methods, using the following formula: $RWC = 100x(FW - DW)/(TW - DW)$. For this, the fresh weight (FW) of a leaf was determined at the harvest. The turgid weight (TW) was calculated in the same leaf after 24 h incubation in deionized water and stored in dark at 4 °C. The dry weight (DW) was subsequently obtained by drying the tissue for at least 48 h in an oven at 80 °C to constant weight.

The leaf water potential (Ψ_w) was determined with a Scholander chamber following the procedure described by Scholander et al. (1965). The leaf water potential measurements were carried out at predawn (Ψ_{wpd}) and six hours after the start of the photoperiod at midday (Ψ_{wmd}).

The osmotic potential (Ψ_o) was measured in the same leaf used for the water potential determination with an osmometer (Osmomat 030, Gonotec, Germany), following the procedure for the freezing point of cellular sap. The osmometer was calibrated with a standard solution of NaCl of 0.3 osmol kg^{-1}. The osmotic potential was calculated according to Wyn Jones and Gorham (1983) by the van't Hoff equation: $\Psi_o = -nxRxT$, where n, R and T denote the concentration (osmol kg^{-1}), ideal gas constant (0.083 kg MPa mol^{-1} K^{-1}) and temperature (K) of the sample, respectively. The leaf osmotic potential measurements were carried out at predawn (Ψ_{opd}) and six hours after the start of the photoperiod at midday (Ψ_{omd}).

The turgor potential (Ψ_p) was calculated as the difference between the leaf water potential and the leaf osmotic potential. The values of the turgor potential were obtained at the same time intervals as in the case of water and osmotic potential: predawn (Ψ_{ppd}) and six hours after the start of photoperiod (Ψ_{pmd}).

Following the same procedure as for Ψ_o, the leaf osmotic potential at full turgor (Ψ_o^{100}) was measured. To obtain full turgor, the leaves were cut and incubated 24 h in deionized water and stored in dark at 4 °C to prevent the loss of dry mass by respiration and the synthesis of new dry mass by photosynthesis. Dehydration (DH) was calculated with the following formula: $DH = \Psi_{omd} - \Psi_o^{100}$.

Osmotic adjustment (OA) was calculated as the difference between the Ψ_o^{100} of the well-watered plants and the Ψ_o^{100} of the drought plants.

The estimated osmotic contribution of each osmolyte to Ψ_o^{100} was obtained using the van't Hoff equation (Tattini et al., 2002). Osmolytes were assumed to have ideal behavior (Alarcón et al., 1993). $\Psi_{osol} = -0.002479xRDWxC$, where Ψ_{osol} indicates the contribution in MPa of individual osmolytes to Ψ_o^{100}; RDW is the relative dry weight at saturation (kg m^{-3}), determined using the equation $RDW = DW/(TW - DW)$, where DW and TW are dry and turgid weights of the sampled leaf; C is the molar concentration of the solute (mol kg^{-1}); and 0.002479 m^3 MPa mol^{-1} is the RT value at 25 °C.

For the determination of proline, aliquots of 20 mg of lyophilized leaf tissue were homogenized with 2 mL aqueous sulfosalicylic acid 3% (Bates et al., 1973). The homogenates were centrifuged at 16,100 *g* for 5 min and the supernatant was kept on ice. To 0.75 mL of the supernatant, 0.75 mL of ninhydrin acid, consisting of 1.25 g of ninhydrin dissolved in 20 mL of 6 M phosphoric acid and 30 mL of glacial acetic acid, was added. Subsequently, to the mixture of the supernatant and ninhydrin acid, 0.75 mL of glacial acetic acid was added. It was incubated for 1 h at 100 °C. Once the tubes cooled in an ice bath, 1.5 mL of toluene was added and shaken vigorously for 20 s. The chromophore-containing toluene was aspirated from the aqueous phase, warmed to room temperature, and the absorbance read at 517 nm (Bates et al., 1973).

Soluble sugars (fructose, glucose, and sucrose) were extracted from freeze-dried leaf tissue (5 mg) in a hydroalcoholic extraction following the procedure described by Pérez-López et al. (2012). It started with the addition of 1 mL of ethanol (80%) and the incubation for 1 h at 4 °C, shaking every 15 min. After centrifugation (13,200 *g* for 5 min), the supernatant was collected. The pellet was re-extracted twice, first with ethanol (60%) and then with milli-Q (MQ) water. The supernatants were combined and the suspension was vacuum dried at 45 °C for 2 h (SPD 121P, Speed-Vac, Thermo Savant, NY, USA). The residue was resuspended in 500 µL of MQ water. Soluble sugars were determined enzymatically using a commercial preparation (Boehringer Mannheim/R-Biopharm Enzymatic Bio Analysis Kit).

For the mineral nutrients determination, dry material was milled with a vibration mill (Model MM301, Fisher Bioblock Scientific) with a continuous shaken 30 s^{-1} for 3 min. Subsequently, calcium (Ca), magnesium (Mg), potassium (K), and phosphorus (P) were quantified using inductively coupled plasma atomic emission spectroscopy (ICPAES; Yobin Yvon Activa, Horiba, Edison, NJ, USA). Cu, Zn, Na, Mn, B and Fe were determined by inductively coupled plasma mass spectrometry (ICPMS, Agilent 7700).

The volumetric elasticity modulus (ε) was estimated assuming a near linear relationship between turgor potential and RWC using the method of Rivelli et al. (2002): $\varepsilon = \Delta P/(\Delta V/V)$, where ΔP is the variation in turgor potential between growth conditions and full turgor conditions: $\Delta P = (-\Psi_o^{100} = \Psi_p^{100}) - \Psi_p$, and ΔV/V is approximated from values for the difference in RWC between fresh and fully hydrated tissue (RWC^{100}): $\Delta V/V = (RWC^{100} - RWC)/100$.

Whole-plant transpiration was calculated by gravimetric method. Each pot was weighed daily at the same time, before and after watering (De Luis et al., 1999). Thus, the water loss in 24 h per pot was obtained.

Hydraulic conductance (HC) was estimated for individual plants by dividing the whole-plant transpiration per area and per day (T), by the difference between the measured predawn leaf water potential (Ψ_{wpd}) minus the midday leaf water potential (Ψ_{wmd}): $HC = T/(\Psi_{wpd} - \Psi_{wmd})$ (Pérez-López et al., 2009).

2.3. Growth parameters

Plants were harvested and separated into shoots and roots, and the samples were dried at 80 °C for 48 h; then, the dry weight was determined. The leaf area was determined using Winfolia software (Regent Instruments Inc., Canada) associated to a scanner (Epson expression 10000 XL).

2.4. Hemicellulose quantification

The samples were analyzed for neutral detergent fiber and acid detergent fiber using a near infrared reflectance spectroscopy instrument (FT-NIR MPA model; Bruker Scientific Instrument, Billerica, MA, USA). Hemicellulose concentration was calculated as the difference between neutral and acid detergent fiber.

2.5. Statistical analysis

Results are reported as the mean ± standard error (SE) of four independent experiments (four experiments under ambient CO_2 and

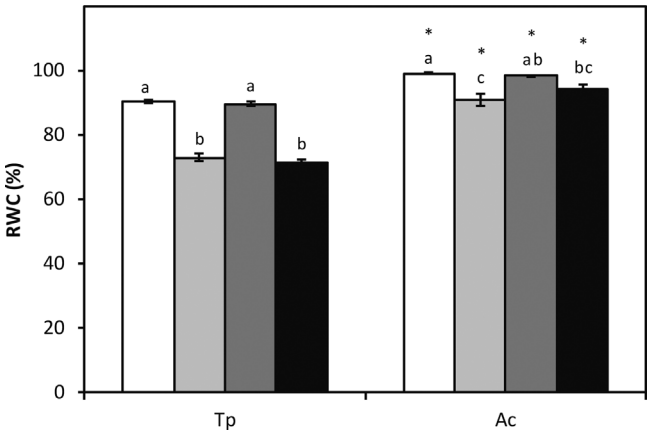

Fig. 1. Effects of drought and CO_2 on relative water content (RWC). White bars represent well-watered plants at ambient CO_2, light gray bars represent drought plants at ambient CO_2, dark gray bars represent well-watered plants at elevated CO_2, and black bars represent drought plants at elevated CO_2. Tp denotes *Trifolium pratense* and Ac denotes *Agrostis capillaris*. Different letters indicate significant differences at $P \leq 0.05$ within the same species. * indicates differences between species.

four experiments under elevated CO_2). In each experiment, we measured at least three different replicates (plants). At each CO_2 concentration, each of the measured physiological parameters remained statistically similar between the four independent experiments; therefore, we pooled the measurements from the four experiments in each condition. Data analyses were performed using the SPSS 19.0 software package (Chicago, IL). Two-way analysis of variance (ANOVA) was used to evaluate the main effects of drought, CO_2 concentration, and their interactions on all dependent variables. Means were compared using Duncan's multiple range test. *P*-values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Leaf water status

After 16 days of withholding water, all treatments in drought conditions presented values of θv *circa* 15%, affecting RWC; however, differences in the behavior of the species were observed (Fig. 1). In Tp, the RWC dropped from 90% in well-watered plants to 70–75% regardless of the concentration of CO_2. In Ac, in which control values were 98%, the decrease in RWC was less than in Tp, reaching values of 91% and 95% at ambient and elevated CO_2, respectively.

Under well-watered conditions, the elevated CO_2 did not change the values of water, osmotic or turgor potential in either species, either at predawn or at midday (Fig. 2). After 16 days of drought, the Ψ_{wpd} of Tp decreased to −0.75 MPa at ambient CO_2, and at elevated CO_2 it diminished to −1.10 MPa (Fig. 2A). In turn, the Ψ_{wmd} of Tp decreased to −1.80 MPa regardless of the concentration of CO_2 (Fig. 2B). In Ac at ambient CO_2, the Ψ_{wpd} was reduced to −0.70 MPa (Fig. 2A) and the Ψ_{wmd} reduced to −1.50 MPa (Fig. 2B). The elevated CO_2 partially mitigated the effect of drought, detecting values of −0.53 MPa (Fig. 2A) and −1 MPa (Fig. 2B) at predawn and midday, respectively.

The Ψ_{opd} of Tp dropped to −1.55 MPa at ambient CO_2 and to −1.70 MPa at elevated CO_2 (Fig. 2C). The Ψ_{omd} decreased to −1.85 and −2.10 MPa at ambient and elevated CO_2, respectively (Fig. 2D). In Ac, no differences were observed (Fig. 2C-D). Regarding turgor potential, the Ψ_{ppd} of Tp was not modified by drought (Fig. 2E); however, the Ψ_{pmd} decreased from 0.75 to 0.09 MPa at ambient CO_2 (Fig. 2F). When the plants grew at elevated CO_2, the decline was smaller, to 0.34 MPa. In contrast to Tp, in Ac drought affected Ψ_{ppd} (Fig. 2E) as well as Ψ_{pmd} (Fig. 2F). Under ambient CO_2, the Ψ_{ppd} and Ψ_{pmd} reached values of 0.54 and 0.01 MPa, respectively. The elevated CO_2 partially alleviated the drop of Ψ_{ppd} and Ψ_{pmd}, to 0.69 and 0.30 MPa, respectively. Overall, the Ψ_{pmd} was higher in Tp than in Ac regardless of the treatment.

3.2. Osmotic adjustment, dehydration and volumetric elasticity modulus

Tp reduced (more negative) its Ψ_o^{100} after 16 days of drought (Table 1). At ambient CO_2, the values of Tp were on average −1.26 MPa, while at elevated CO_2 the values were 6% more negative (−1.34 MPa). In contrast, in Ac, it was not affected. These lower Ψ_o^{100} values resulted in OA in Tp, but not in Ac. The OA in Tp was dependent on the concentration of CO_2, OA being 20% higher at elevated CO_2. Drought increased the DH of Tp at ambient CO_2 to 0.58 MPa and to 0.76 MPa at elevated CO_2. In Ac, DH was lower than in Tp (0.19 MPa) under both CO_2 concentrations.

Another mechanism that can also contribute to drought tolerance is based on adjustments of cell wall elasticity. Elastic adjustment of the cell wall may alter the relationship between cell volume and turgor and thus allows plants to better cope with drought. ε was higher in Ac than in Tp (Table 1). It is striking that ε responded in a different way to water suppression in each species. Drought caused an increase in the ε of Tp of 2–3 times compared to plants grown under well-watered conditions regardless of the concentration of CO_2. In contrast, in Ac, drought reduced ε by 85% and 65% under ambient and elevated CO_2, respectively.

The properties of the cell wall are determined by the influence of its carbohydrate polymers, pectin, cellulose, and, in particular, the xyloglucans and xylans of hemicellulose, on ε. In both species, a negative correlation between the concentration of hemicellulose and ε was detected, independent of the concentration of CO_2 (Fig. 3); the higher the concentration of hemicellulose the greater the cell wall's elasticity (lower ε).

On the other hand, a wide range of substances can contribute to OA, including inorganic cations and anions, organic acids, carbohydrates, and amino acids, which lead to a more negative osmotic potential. In this respect, on day 16, under well-watered conditions and at ambient CO_2 the concentration of soluble sugars was 65% higher in Ac than in Tp (Fig. 4A). The elevated CO_2 increased the concentration of soluble sugars in Tp by 21%. After 16 days of drought at ambient CO_2, the concentration of sugars in Tp increased by 63%, while at elevated CO_2 the increases were of 70%. In Ac, drought did not produce significant differences in the concentration of soluble sugars regardless of the concentration of CO_2.

After 16 days of drought under ambient CO_2, the proline concentration in Tp was approximately 20 times higher compared to plants under well-watered conditions (Fig. 4B). When Tp was grown at elevated CO_2, the increase was greater, the proline concentration rose approximately 60 times in drought plants. In contrast, in the case of Ac, no differences in the concentration of proline between treatments were detected.

The contribution of solutes to Ψ_o^{100} is shown in Table 2. The largest contribution, expressed as a percentage, was from inorganic ions in both species, being close to 60% under well-watered conditions. Among inorganic ions, K^+ was the cation that in percentage terms contributed more to the osmotic potential at full turgor: 32% and 45% in Tp and Ac, respectively. The organic compounds analyzed were approximately 5% in Tp and 6% in Ac. The elevated CO_2 increased the contribution of organic solutes in Tp, up to 6% due to an increase in the contribution of sugars.

After 16 days of drought, the contribution of inorganic ions to Ψ_o^{100} in Tp was not affected at ambient CO_2 (Table 2). However, the organic compounds doubled their contribution, up to 11%. If the contribution of the organic compounds is broken down, it can be seen that sugars increased to 8% and proline to 3%. Under combined conditions of elevated CO_2 and drought, the contribution of organic compounds analyzed rose to 19% due to an increase in the contribution of sugars and proline, reaching 10.5% and 8.5%, respectively.

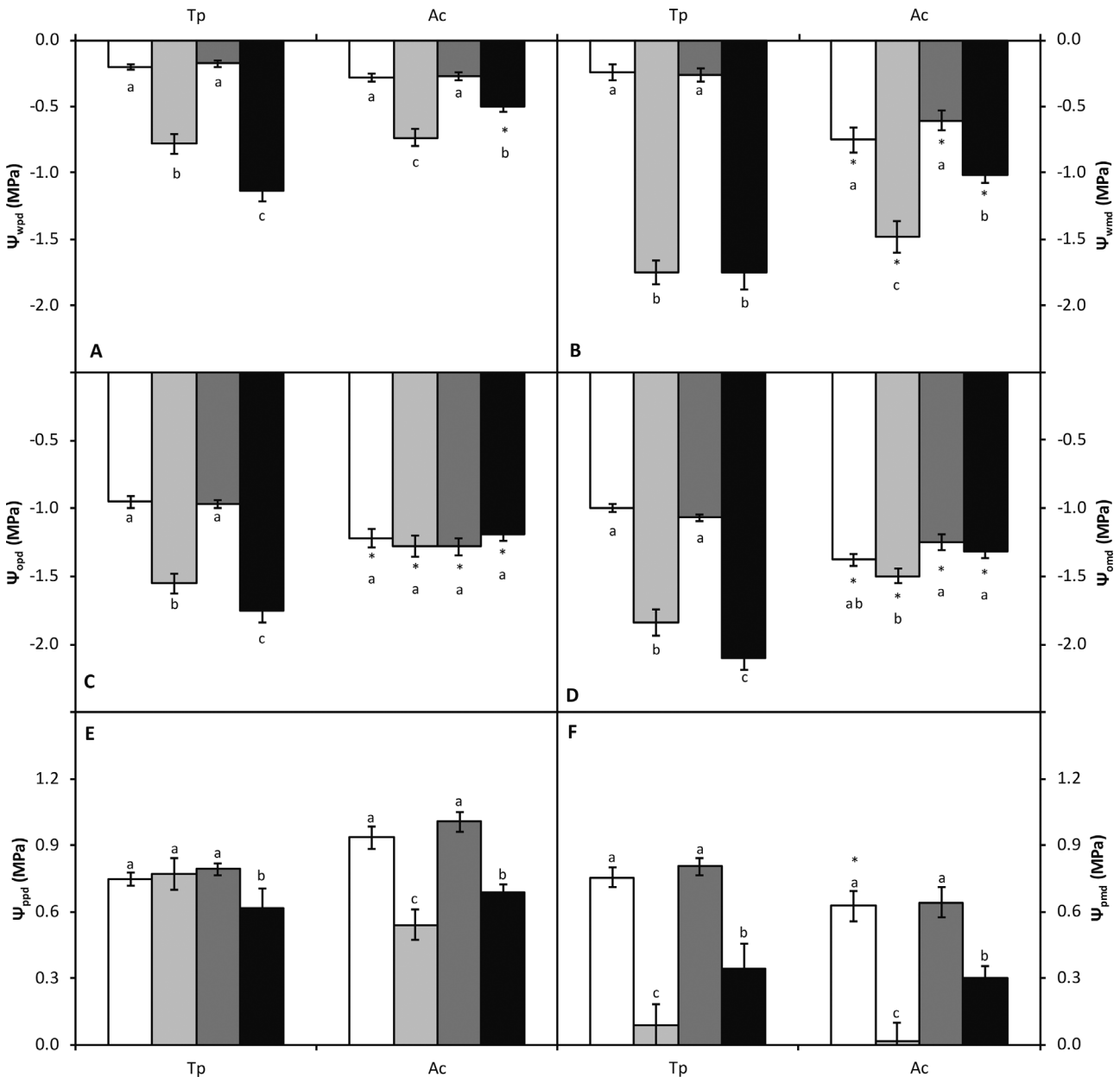

Fig. 2. Effects of drought and CO_2 on leaf predawn water potential (Ψ_{wpd}; A), leaf midday water potential (Ψ_{wmd}; B), leaf predawn osmotic potential (Ψ_{opd}; C), leaf midday osmotic potential (Ψ_{omd}; D), leaf predawn turgor potential (Ψ_{ppd}; E), and leaf midday turgor potential (Ψ_{pmd}; F). Growth conditions and statistical analysis, as depicted in Fig. 1.

Table 1
Effects of drought and CO_2 on osmotic potential at full turgor (Ψ_o^{100}; MPa), osmotic adjustment (OA; MPa), dehydration (DH; MPa), and volumetric elasticity modulus (ε; MPa). Tp denotes *Trifolium pratense*; Ac, *Agrostis capillaris*; D, drought; WW, well-watered. Different letters indicate significant differences at $P \leq 0.05$ within the same species. * indicates differences between species.

	Tp				Ac			
	400 μmol mol^{-1}		700 μmol mol^{-1}		400 μmol mol^{-1}		700 μmol mol^{-1}	
	WW	D	WW	D	WW	D	WW	D
Ψ_o^{100}	−0.90 ± 0.04 b	−1.26 ± 0.04 a	−0.91 ± 0.11 b	−1.34 ± 0.04 a	−1.27 ± 0.07 a*	−1.30 ± 0.05 a	−1.30 ± 0.09 a*	−1.14 ± 0.03 a
OA	–	0.36 ± 0.06 a	–	0.43 ± 0.13 a	–	0.03 ± 0.08 a*	–	−0.16 ± 0.06 a*
DH	0.10 ± 0.04 b	0.58 ± 0.07 a	0.16 ± 0.11 b	0.76 ± 0.09 a	0.11 ± 0.05 a	0.19 ± 0.05 a*	−0.05 ± 0.04 b	0.18 ± 0.05 a*
ε	1.49 ± 0.46 b	4.36 ± 0.15 a	1.70 ± 0.88 b	3.48 ± 0.14 a	88.11 ± 9.31 a*	14.33 ± 0.56 c*	42.53 ± 5.49 b*	14.47 ± 0.51 c*

In Ac, at ambient CO_2, drought did not modify the contribution pattern (Table 2). At elevated CO_2, the contribution of inorganic ions increased to 63%. The increase in inorganic ions was mainly due to an increase in the contribution of K^+ but also due to a slight increase in the contribution of Ca^{2+} and Mg^{2+}. The contribution of organic solutes grew to 8.6%; the increase was provoked by the increase in sugars.

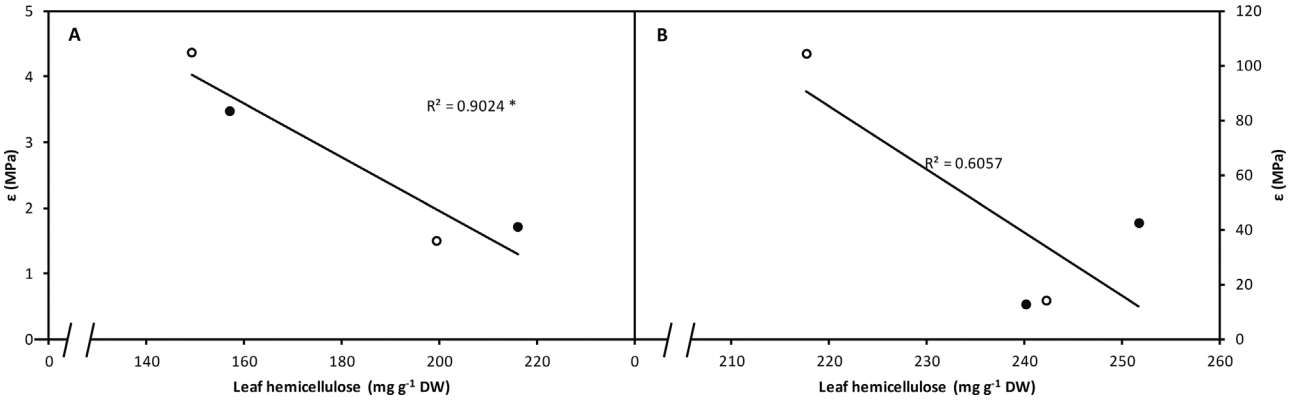

Fig. 3. Relation between leaf hemicellulose concentration and volumetric elasticity modulus (ε) in Tp (A) and in Ac (B) grown at ambient (open symbols) or elevated (closed symbols) CO_2 concentration. No significant differences between CO_2 concentrations were observed. The symbols used *, **, *** indicate significant correlation at 5%, 1% and 0.1%, respectively.

3.3. Leaf area, root to shoot ratio, transpiration and hydraulic conductance

The leaf area of Tp was 2.5 times higher than the one of Ac under well-watered conditions and ambient CO_2 (Fig. 5A). The elevated CO_2 increased the leaf area of both species *circa* 20%. Under drought conditions the leaf area of both species was similar, since the decreases in Tp were higher than in Ac, 70% and 27%, respectively, regardless of the CO_2 concentration.

Both species showed a similar value of root to shoot ratio under well-watered conditions and ambient CO_2 (Fig. 5B). However, the effect of elevated CO_2 and drought on root to shoot ratio was species-specific. Elevated CO_2 increased the ratio in Ac, but not in Tp. Drought increased root to shoot ratio in Tp by 54% under ambient CO_2 and by 74% under elevated CO_2, whereas in Ac, drought increased the ratio under ambient CO_2 and it remained constant at elevated CO_2.

On day 16, Tp presented the highest values of cumulative transpiration per pot, and those of Ac were 60% lower (Fig. 5C). In well-watered conditions, the elevated CO_2 increased the cumulative transpiration by 15% in Tp, but not significantly in Ac. Water stress at ambient CO_2 caused a decrease of 56% in the cumulative transpiration per pot in Tp. At elevated CO_2, the decline in cumulative transpiration per pot was higher (62%). However, in Ac, no differences were observed at either concentration of CO_2.

The HC was, generally, lower in Ac than in Tp (Fig. 5D). The elevated CO_2 decreased HC under well-watered conditions in Tp but not in Ac. Drought reduced HC in all treatments, more notably in Tp than in Ac. In the case of Tp, the water stress reduced the HC by approximately 95% regardless of the concentration of CO_2. In Ac, the declines were 53% and 18% at ambient and elevated CO_2, respectively.

4. Discussion

4.1. Effects of drought under ambient CO_2

The soil volumetric water content was manipulated so all treatments presented the same soil water content; however, differences in the response to this soil water content between species were observed. Ac showed a higher RWC than Tp (Fig. 1); as the RWC is a balance between water uptake and loss, one would expect differences in any of these two processes. As noted previously, to avoid a massive fall in RWC, the plant can, on one hand, avoid the decrease in water uptake and, on the other hand, minimize water loss through transpiration. Indeed, lower stomatal conductance was observed in Ac compared with Tp (data not shown).

To avoid the decrease in water uptake, the plant has mainly two options: to increase carbon allocation to roots and to diminish its water potential (Pérez-López et al., 2009; Xu et al., 2013). Both species increased their root to shoot ratio. However, the response of root to shoot ratio to drought was different between species (increasing more in Ac than in Tp), remarking that some responses or the magnitude of those responses are species-specific (Xu et al., 2014). Since the increase in the root to shoot ratio has been signaled as a drought tolerance mechanism because it enhances the soil exploration and permits higher water uptake (Markesteijn and Poorter, 2009), our results would indicate that Ac was more drought tolerant than Tp under ambient CO_2.

Regarding the other option, one of the mechanisms by which plants achieve the decrease in water potential is lowering the osmotic potential. Several authors have argued that the plant reduces its osmotic potential only when its survival is at risk (Serraj and Sinclair, 2002), which concurs with our results since a decrease in osmotic potential

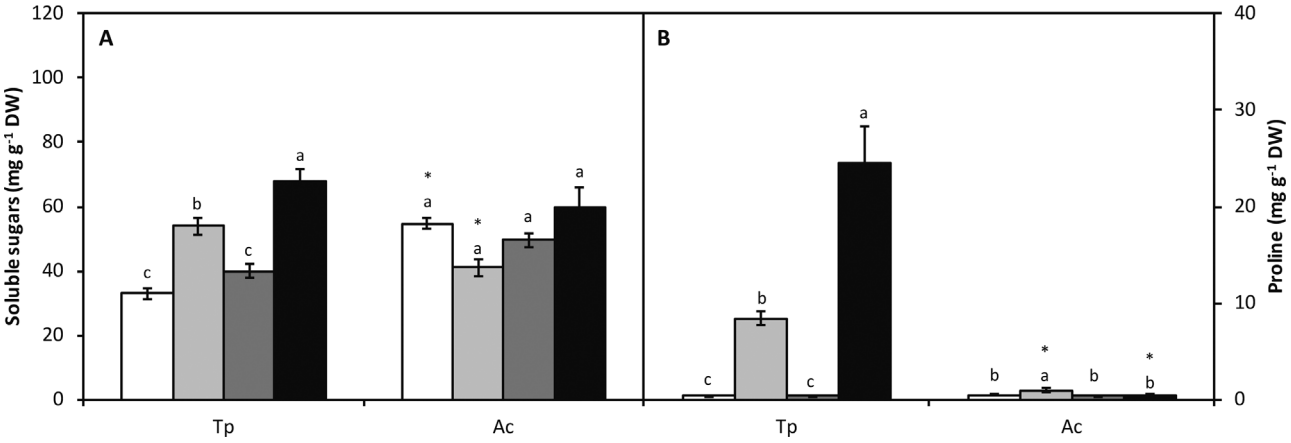

Fig. 4. Effects of drought and CO_2 on soluble sugars concentration (A) and proline concentration (B). Growth conditions and statistical analysis, as depicted in Fig. 1.

Table 2
Effects of drought and CO_2 on the contribution of solutes (MPa) to the Ψ_o^{100} in Tp and in Ac; in brackets the percentage of contribution of each solute. Tp denotes *Trifolium pratense*; Ac, *Agrostis capillaris*; D, drought; WW, well-watered.

		400 μmol mol^{-1}				700 μmol mol^{-1}			
		WW		D		WW		D	
Ψ_o^{100}	Tp	−0.90		−1.26		−0.91		−1.34	
	Ac	−1.34		−1.30		−1.30		−1.14	
K^+	Tp	−0.29	(32.5)	−0.39	(30.6)	−0.25	(27.5)	−0.33	(24.8)
	Ac	−0.60	(44.8)	−0.60	(46.2)	−0.65	(49.8)	−0.58	(50.6)
Ca^{2+}	Tp	−0.19	(20.6)	−0.26	(20.8)	−0.19	(20.6)	−0.33	(24.6)
	Ac	−0.04	(3.3)	−0.05	(3.8)	−0.05	(4.0)	−0.05	(4.7)
Mg^{2+}	Tp	−0.09	(9.8)	−0.14	(10.0)	−0.09	(10.0)	−0.17	(12.4)
	Ac	−0.07	(5.3)	−0.08	(5.8)	−0.09	(6.7)	−0.08	(6.7)
Inorganics	Tp	−0.57	(63.5)	−0.80	(63.5)	−0.53	(58.4)	−0.83	(62.2)
	Ac	−0.72	(53.9)	−0.74	(57.0)	−0.80	(61.2)	−0.72	(63.3)
Sugars	Tp	−0.04	(4.60)	−0.10	(8.25)	−0.05	(5.95)	−0.14	(10.38)
	Ac	−0.08	(5.80)	−0.07	(5.54)	−0.08	(6.03)	−0.10	(8.45)
Proline	Tp	−0.00	(0.15)	−0.04	(2.88)	−0.00	(0.15)	−0.12	(8.58)
	Ac	−0.00	(0.14)	−0.00	(0.29)	−0.00	(0.15)	−0.00	(0.18)
Organics	Tp	−0.04	(4.74)	−0.14	(11.13)	−0.06	(6.10)	−0.25	(18.96)
	Ac	−0.08	(5.94)	−0.08	(5.83)	−0.08	(6.18)	−0.10	(8.64)
Other solutes	Tp	−0.29	(31.8)	−0.32	(25.3)	−0.32	(35.5)	−0.25	(18.9)
	Ac	−0.54	(40.1)	−0.48	(37.2)	−0.42	(32.6)	−0.24	(28.1)

Inorganics is the sum of all the inorganic ions measured. Other solutes (unknown) were calculated as the difference between Ψ_o^{100} and the sum of calculated osmotic components of individual solutes.

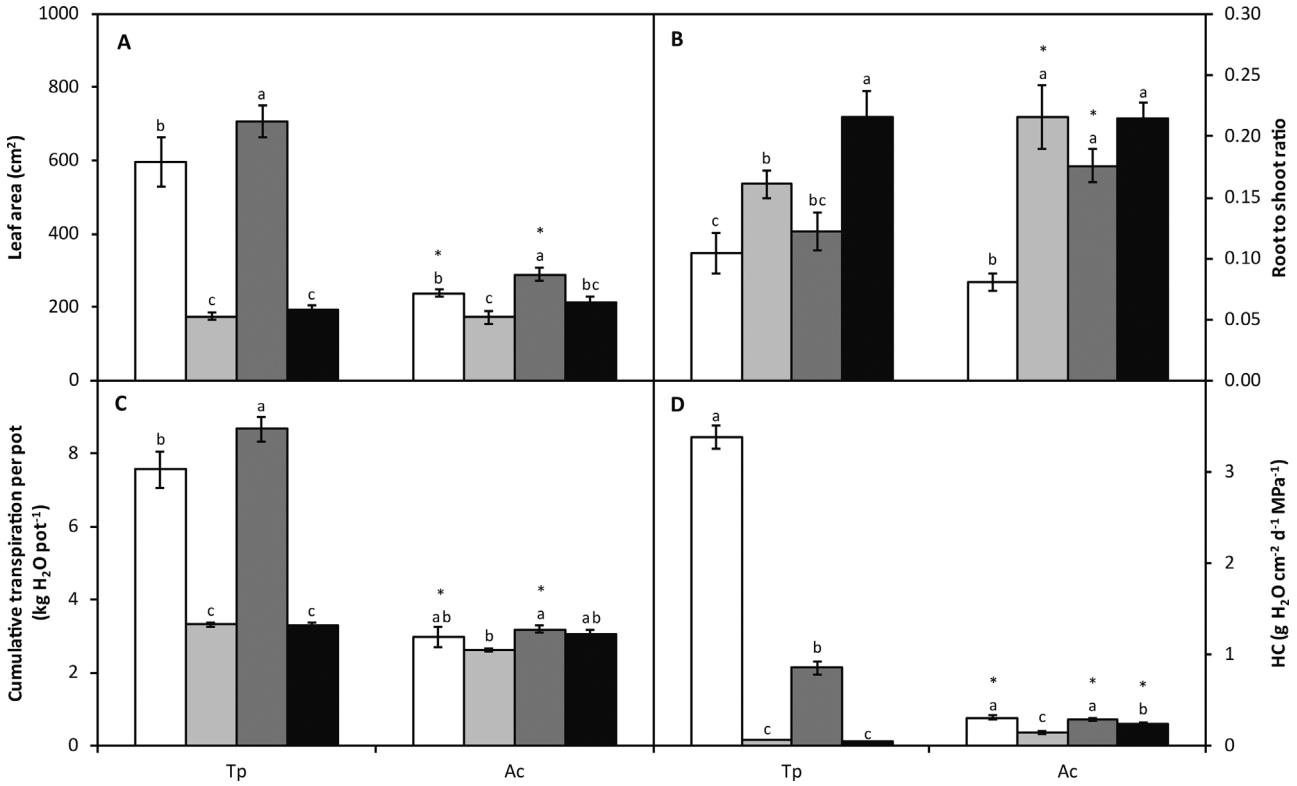

Fig. 5. Effects of drought and CO_2 on leaf area (A), root to shoot ratio (B), cumulative transpiration per pot (C), and hydraulic conductance (HC; D). Growth conditions and statistical analysis, as depicted in Fig. 1.

was only observed in the species more affected by drought, *i.e.*, in Tp (Fig. 2D).

This decrease in osmotic potential can be caused by dehydration (having less water in the cells provokes the concentration of solutes to rise) or by active synthesis of osmolytes, what is known as osmotic adjustment. In Tp, the decrease in osmotic potential was due to both processes (Table 1). On one hand, Tp induced the synthesis of compatible osmolytes (proline and sugars, Fig. 4), on the other hand, the concentration of osmolytes also increased through a concentration effect from dehydration; the effect of dehydration was approximately 60% higher than the active synthesis of osmolytes (Table 1). In Ac, osmotic adjustment was not observed when the RWC decreased to 90%; however, other authors observed osmotic adjustment in plants of the genus *Agrostis* despite the RWC having decreased only to 90% (DaCosta and Huang, 2006), indicating a species-specific response.

Other authors have also demonstrated the synthesis of proline and sugars as compatible molecules in grassland species: Iannucci et al. (2002) and Lee et al. (2008) in clover and DaCosta and Huang (2006) in

plants of the genus *Agrostis*. Other compounds such as γ-aminobutyric acid, polyamines (De Diego et al., 2013), glycinebetaine (Silva et al., 2010), and various polyalcohols such as mannitol and sorbitol (Bohnert et al., 1995) have also been proposed as organic compounds that plants may synthesize for osmotic adjustment, so a contribution of organic compounds higher than the one detected cannot be ruled out, and in this case it would be encompassed in the component called "other solutes" (Table 2).

As aforementioned, the decrease in the osmotic potential allowed the plant to reduce its water potential to maintain water uptake. However, in all cases in which passive dehydration was involved as a mechanism for lowering the osmotic potential, the turgor potential was reduced due to the lower water content in the cells, indicating that the osmotic adjustment was not enough to maintain water status.

Despite the fact that dehydration occurs, to prevent a massive decrease in turgor potential, the plant alters its cell wall elasticity, making it more elastic (Kozlowski et al., 1991). This increased cell wall elasticity –decreased ε– delays the loss of turgor with a reduction of water potential (Sanders and Arndt, 2012). However, there have also been cases where a plant increases the rigidity of the cell wall to reduce water potential with a minimum loss of water content in the plant and thereby maintain water uptake (Kozlowski et al., 1991; Touchette et al., 2007). In this case, the symplast volume is maintained at the expense of turgor (Sanders and Arndt, 2012, and references therein). Both processes are considered advantageous for the plant (Schulte, 1992; Saito and Terashima, 2004). The rigidity of the cell wall, as occurred for OA, also followed different trends depending on the species. In Tp, the ε increased significantly (Table 1), implying that Tp tries to maintain water uptake at the expense of reducing turgor (Fig. 2F). Conversely, Ac showed an opposite tendency from Tp, *i.e.*, ε decreased. However, although it did not show high dehydration, the turgor potential was reduced to values close to 0. This could be caused by the high rigidity of the cell wall (despite having decreased its rigidity in drought), which implies that a small change in RWC is translated into a high decrease in turgor potential, although the value of RWC was up to 90%.

These facts confirm what it is seen in the literature, *i.e.*, the response of cell wall elasticity to drought is species-specific, showing increases as well as decreases in grassland species (Barker et al., 1993; Chai et al., 2010). This different strategy between species could indicate that the survival of Tp was threatened and it therefore makes the cell wall more rigid so that at the expense of turgor potential, and therefore of growth, it could maintain water uptake.

The variation in the volumetric elasticity modulus in drought has been associated, among other factors, with changes in the components of the cell wall, the appearance of cell wall proteins and the boron concentration (Findeklee and Goldbach, 1996; Marshall and Dumbroff, 1999). A correlation with boron was not detected in either species (data not shown); however, a negative correlation with the concentration of hemicellulose was observed (Fig. 3), *i.e.*, the higher the concentration of hemicellulose the higher the cell wall elasticity, confirming that changes in the composition of the cell wall could be responsible for the change in elasticity of the cell wall. It has been stated that hemicellulosic polysaccharides, which form bridges between the microfibrils of cellulose, are responsible for controlling the elasticity of the cell wall. Xylan and 1,3-1,4-β-glucan are the main hemicellulosic polysaccharides in poaceae such as Ac, while in dicot plants, as in Tp, xyloglucan is the major hemicellulosic polysaccharide that contributes to cell wall elasticity (Takahashi et al., 2015). These differences in the polysaccharides might be the cause of the different ε behavior between our species.

Additionally, to minimize water loss by transpiration, the plants reduced their leaf area (Fig. 5A) and hydraulic conductance (Fig. 5D). Both species reduced leaf area aiming to decrease water loss by transpiration, as a drought tolerance mechanism (Cattivelli et al., 2008). Although no differences were detected in the absolute values between species, the decrease was higher in Tp than in Ac, indicating that this tolerance mechanism was of higher magnitude in the former than in the latter. On the other hand, regarding hydraulic conductance, various causes have been described for the change in hydraulic conductance, including stomatal closure, a change in activity of aquaporins, and cavitation (Tyree and Sperry, 1989; Cochard et al., 2002; Kaldenhoff et al., 2008). In general, in nearly all treatments, the percentage reduction in stomatal conductance (data not shown) was lower than the decrease in hydraulic conductance (Fig. 5D); therefore, the presence of other mechanisms such as those mentioned above cannot be ruled out for these species.

The first hypothesis was that the species that can develop more efficient mechanisms of drought tolerance will present greater turgor potential and leaf water content. Both species activated mechanisms of drought tolerance, although the mechanisms were different between species; however, both species showed values of turgor potential close to 0 MPa, so we could not confirm the first hypothesis. These findings indicate that these tolerance mechanisms are not enough to maintain turgor potential and leaf water content.

4.2. Effects of drought under elevated CO_2

As stated in the introduction, several authors have observed that elevated CO_2 partially mitigated the negative effects of drought on plant water relations (Samarakoon and Gifford, 1995; Morgan et al., 2004; Robredo et al., 2007). In the literature cited above, the soil water content was different between CO_2 concentrations, which would imply that an indirect effect of CO_2 was observed, *i.e.*, the elevated CO_2 improves plant water relations through higher water soil conservation. Since in this study the soil water content was similar between CO_2 concentrations, it would allow to erase the indirect effect of elevated CO_2 on plant water relations through higher soil water conservation, permitting the possibly direct effects of elevated CO_2 on plant water relations to be detected.

As mentioned, all treatments presented the same soil volumetric water content as at ambient CO_2; in addition, there were no differences between CO_2 treatments in the RWC (Fig. 1). In Tp, differences in Ψ_{wmd} were not found between CO_2 treatments (Fig. 2B); although elevated CO_2 did not affect the water potential of Tp, the elevated CO_2 partially mitigated the negative effect of drought on the turgor potential of Tp (Fig. 2F), possibly because under elevated CO_2, Tp increased even more the root to shoot ratio in response to drought than under ambient CO_2. This was, possibly, caused by the increased availability of carbon skeletons as a result of the higher photosynthetic rates at elevated CO_2 than at ambient CO_2 in drought conditions. Under elevated CO_2, a higher carbohydrate proportion is being deviated to roots to cope with water stress as was also demonstrated by Xu et al. (2007), indicating that elevated CO_2 will be favoring this tolerance mechanism. Besides, a 20% higher OA was found at elevated CO_2 than at ambient CO_2 (Table 1) which would also explain the partial mitigation of the negative effect of drought on the turgor potential of Tp.

Several authors have reported that, at elevated CO_2, plants would present greater OA due to the increased availability of carbon skeletons, as aforementioned due to the higher photosynthetic rates at elevated CO_2 (Ferris and Taylor, 1994; Wall et al., 2006; Chen et al., 2015), as is the case in Tp (data not shown), which increased the synthesis of sugars and proline (Fig. 4). The higher decrease in the osmotic potential enabled the plant to maintain a higher turgor potential. This would confirm the second hypothesis that elevated CO_2 will partially mitigate the negative effects of drought through higher osmotic adjustment and root to shoot ratio and not through higher soil water conservation.

However, other authors have not observed differences in osmotic adjustment despite having higher photosynthetic rates at elevated CO_2 (Tschaplinski et al., 1995; Robredo et al., 2007), as is the case in Ac (Table 1), which implies that the second hypothesis is not fulfilled for Ac. However, in Ac, elevated CO_2 also mitigated the negative effects of drought because the turgor potential was higher at elevated CO_2, indicating that other mechanisms, and not only OA and root to shoot

ratio, should be working at elevated CO_2. This difference in turgor potential between CO_2 concentrations might be due to the high rigidity of the cell wall (Table 1), so a small change in the RWC permits it to maintain the turgor potential higher than at ambient CO_2; although the difference in RWC between CO_2 concentrations was not statistically significant, the small increase in RWC could have allowed the plant to have a higher turgor potential (Saito and Terashima, 2004).

The greater RWC in Ac might also be explained by the higher HC (Fig. 5D), which was 63% higher at elevated CO_2 than at ambient CO_2, which would allow the plant to uptake more water. This increase in HC at elevated CO_2 might be explained by higher xylem conductance as proposed by Heath and Kerstiens (1997). Additionally, gs decreased more than HC under elevated CO_2 conditions (48%), which implies that the end of the bulk flow of water is more restricted than the start, permitting retention of higher water content. Therefore, the positive effect of elevated CO_2 relies not only on a higher OA but also on changes in HC.

In conclusion, the species counteracted drought through different mechanisms. Tp performed OA and increased the root to shoot ratio and the rigidity of the cell wall in order to be able to maintain water uptake. In contrast, Ac did not make OA but decreased the rigidity of the cell wall to avoid a massive fall in turgor potential due to the loss of water content. Besides, it also increased root to shoot ratio. However, both species showed values of turgor potential close to 0 MPa, indicating that these mechanisms were not sufficient to counteract all the negative effects of drought stress. Under combined conditions and when the soil water content was similar between CO_2 concentrations, the elevated CO_2 mitigated the negative effect of drought in both species; however, the mechanism was different. In Tp, thanks to the higher photosynthetic rates under elevated CO_2 conditions, the plant increased its root to shoot ratio and the synthesis of organic compounds (proline and sugars) and increased OA permitting a higher turgor potential. In Ac, the elevated CO_2 provoked a higher HC, which permitted an increase in water uptake, and together with the higher decrease in gs than in HC, maintained a slightly higher RWC, therefore higher turgor potential under elevated CO_2 conditions.

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